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## **Archaea are the predominant and responsive ammonia oxidizing prokaryotes in a red paddy soil receiving green manures**

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2    **a red paddy soil receiving green manures**

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## **Abstract**

Application of green manures is an effective approach to optimizing N management in paddy soils. Nitrification is a key process in the N cycle and ammonia oxidization is the first and typically limiting step in nitrification. In this study, we investigated the changes of ammonium oxidizing prokaryotes after the application of green manure in a red paddy soil using pot experiments. The experiment included four treatments; milk vetch-rice, radish-rice, ryegrass-rice and winter fallow-rice. The nitrification potential was measured, and the abundance and community of *amoA* genes from ammonia-oxidizing archaea (AOA) and bacteria (AOB) were quantified. The results showed that the AOA to AOB ratios ranged from 7 to 80, and that the milk vetch treatment increased the abundances of AOA and AOB. The abundance of AOA showed negative correlations with nitrification potential and  $\text{NH}_4^+\text{-N}$ , and positive correlation with soil pH in the acidic red paddy soil. DNA sequence analyses revealed that the *Nitrososphaera* and *Nitrospira* were the dominant clusters of AOA and AOB, respectively. The dominant clusters of AOA were significantly changed by utilization of green manures, especially radish. Partial least squares path modeling analysis showed that green manures exerted larger effects on the abundances of AOA than on AOB, and the community structure of AOA had the strongest effect on nitrification potential. The high abundance of AOA found in this study and their responsiveness to green manuring suggests that AOA are critically important for soil ammonia oxidation in these soils and more sensitive to green manuring than AOB.

*Key words:* green manure; ammonia-oxidizing archaea; nitrification potential; driving factors; red paddy soil

## 1. Introduction

Nitrification is the conversion of inorganic nitrogen from a reduced form to an oxidized state [1]. Ammonia oxidization is the first and typically the rate-limiting step, carried out by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA). The *amoA* gene, which can occur in both AOA and AOB is a good indicator of the abundance and diversity of ammonia oxidizing prokaryotes, and is commonly used as molecular biomarker in the studies of nitrification [2]. The abundance and communities of AOA and AOB are influenced by soil properties in agricultural soil. Many reports have revealed that pH is the main factor driving the community changes of AOA and AOB [2-6]. Substrate availability such as the amount of ammonia is also an important driver of both AOA and AOB species richness [2, 7, 8]. Other field management practices and soil properties such as fertility regime [9, 10], manure input [11, 12], soil moisture and temperature [13] may also affect the nitrification process and the distribution and activity of ammonia oxidizers. The relative contribution of AOA and AOB in different types of soils are still under debate [10]. Numerous studies have confirmed that AOA are predominate ammonia-oxidizing prokaryotes when compared to their bacterial counterparts in multiple terrestrial environments [14], such as those with alternating oxygenated/hypoxic conditions [15], and acidic soils [16], suggesting that AOA could have a potentially greater role in the overall nitrification than their bacterial counterparts [17].

The utilization of green manures in crop rotations is a management practice designed to improve soil fertility and reduce chemical fertilizer applications [18-20]. In southern China, the planting of winter green manures is used as an effective means of improving the productivity and sustainability of paddy fields under double-rice cropping systems [21, 22], and milk vetch (*Leguminosae*), radish (*Cruciferae*) and ryegrass (*Gramineae*) are popular green manure species in this area. The production practices have proved that all of these three kinds of green manure improved the grain yield and soil fertility, and the effects of these three green manures have differences, but the mechanisms are unclear. The characteristics of the plant residues differ between plant families, and the release of nutrients during decomposition may have various influences on the abundance and diversity of AOA and AOB. Many studies have investigated the ammonia oxidizers in different kinds of paddy soils with various management practices [4, 9, 23, 24]. However, the effects of different green manures on the abundance and community structure of ammonia oxidizers is not well understood. It is possible for us to find out the driving factors in nitrification by evaluating the responses of ammonia oxidizer on different green manures. After incorporation of green manures, the decomposition of plant residues and the priming effects may have more profound influences on soil conditions than the stage after rice harvest. In the period after rice cultivation, the interactive effects of green manure decomposition and rice growth may also change the nitrification process and activity of ammonia oxidizers. So, it is reasonable to compare the variations before and after rice cultivation.

In this study, we investigated the abundance and community diversity of ammonia oxidizers at the stages before rice transplantation and after rice cultivation in the winter green manure – double rice system. We hypothesized that (i) AOA *amoA* gene would be more abundant in this

acidic paddy soil thus indicate the predominant of AOA compared with AOB, (ii) the different green manures had various effects on AOA and AOB, (iii) sampling stage may be one of the driving factors that led to the changes of ammonia oxidizers.

## **2. Materials and methods**

### *2.1 Plant materials and soils*

Pot experiments were conducted in the Red Soil Experimental Station (26°45' N, 111°52' E; elev. 150 m) of Chinese Academy of Agricultural Sciences, Qiyang, Hunan Province, China. The paddy soil is derived from Quaternary red clay, and classified as a Ferralic Cambisol [25]. On October 20, 2013, milk vetch (*Astragalus sinicus* L.), radish (*Raphanus sativus* L.) and ryegrass (*Lolium multiflorum*) were planted in three field plots, with a winter fallow plot as a control (weeds in the winter fallow plot were removed by hand and the plot was kept plant free). Soils and plants were collected at the full-bloom stage of milk vetch and radish (March 25, 2014). The aboveground parts of milk vetch, radish, ryegrass and their corresponding soils, as well as the soil from the winter fallow plots were sampled for the use of following pot experiments. Soils was collected to a depth of 0.2 m and sieved through a 2 mm mesh prior to use in the pot experiment. The properties of green manures and soils are shown in [Tables S1 and S2](#).

### *2.2 Experimental design and sampling*

Four treatments were designed including milk vetch-rice (the milk vetch soil was incorporated with the corresponding milk vetch, MV), radish-rice (the radish soil was incorporated with the corresponding radish, RD), ryegrass-rice (the ryegrass soil was incorporated with the

corresponding milk ryegrass, RG) and winter fallow-rice (the milk vetch soil without plant residues was incorporated, WF). Each treatment was conducted in triplicate with a completely randomized design. The size of pots used in the experiments was 275 mm in height and 270 mm in diameter, and a total of 10 kg soil was packed into each pot. The green manures were collected at their full bloom stage, then the plants were chopped and weighed. The amount of green manure incorporated in each pot was 200 g fresh biomass. Each pot was received 0.6 g N, 0.6 g P<sub>2</sub>O<sub>5</sub> and 0.6 g K<sub>2</sub>O (4 g 15-15-15 NPK compound fertilizer) as a basal fertilizer. All chemical fertilizer and green manures were applied on March 25, 2014. Rice plants were transplanted 30 days after the application of green manures. All pots were flooded after fertilization and during the growth of the rice.

Soils were sampled at two stages: 1) 30 days after the incorporation of green manure (before the rice was transplanted, on 25 April, Stage 1), and 2) after the rice was harvested (on 11 July, Stage 2). After being mixed well and sieved (< 2 mm), soil samples were divided, one batch was then stored at -20°C for DNA extraction, and a second batch was stored at 4°C prior to chemical analyses. After the rice harvest, the grain, shoots and roots were sampled separately, dried and milled to measure the yields and nutrient contents.

### *2.3 Chemical analysis*

All soil analyses were conducted according to Lu [26]. Soil pH was measured using pH electrode with a soil to water ratio of 1:2.5. Soil total nitrogen (TN) was determined using the Kjeldahl method. Soil organic matter (SOM) and total C of the plants were determined with the potassium dichromate oxidation method. Soil available phosphorus (AP) and available potassium

(AK) were extracted by 0.5 mol L<sup>-1</sup> NaHCO<sub>3</sub> and 1 mol L<sup>-1</sup> CH<sub>3</sub>COONH<sub>4</sub> respectively. Soil NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N were extracted by 2 M KCl, and measured on a continuous flow analyzer (AA3, SEAL, Germany).

#### 2.4 Nitrification potential

Nitrification potential (NP) was measured using the chlorate slurry inhibition assay with slight modifications [1]. Briefly, each soil sample (5.0 g) was transferred to a 25 ml centrifuge tube including the 20 ml liquid medium (the concentration of NH<sub>4</sub><sup>+</sup>-N was 100 mg L<sup>-1</sup> and the pH of the medium was adjusted to 7.5 using H<sub>2</sub>SO<sub>4</sub> or NaOH solution). The slurry with soil and liquid medium was incubated for 5h at 25°C, and 0.2 ml sodium chlorate (1 M) was added to inhibit the oxidization of nitrite to nitrate during the incubation. After incubation, 5 ml of 2 M KCl was added to the extract and the nitrite released during the incubation period was then determined on a continuous flow analyzer (AA3, SEAL, Germany).

#### 2.5 DNA extraction and real time quantitative PCR

Three DNA extractions per sample were performed using a FastDNA Spin Kit for Soil (MP Bio, Santa Ana, CA, USA) following the manufacturer's procedures. Three extractions from each soil sample were pooled and DNA was then quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). The DNA samples were stored at -80°C for further analysis.

Real-time quantitative PCR of archaeal and bacterial *amoA* genes were performed on 7500 Real time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, US) in triplicate. The 25 µL



reactions contained the following ingredients: 12.5µL of Power SybrGreen qPCR Master Mix (Thermo Fisher Scientific Inc.), 0.5 µL each of 10 µM forward and reverse primers for ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), 9.5µL of ddH<sub>2</sub>O and 2µL of 10-fold diluted extracted DNA. Cycling protocols were 5 min at 95°C followed by 40 cycles of 5s at 95°C, 30s at 56°C, and 40s at 72°C for both AOA and AOB *amoA* genes. The primers for AOA were Arch-amoAF (STAATGGTCTGGCTTAGACG) and Arch-amoAR (GCGGCCATCCATCTGTATGT) [27], with the amplicon length of 635 bp. The primers for AOB were amoA-1F (GGGGTTTCTACTGGTGGT) and amoA-2R (CCCCTCKGSAAAGCCTTCTTC) [28], with an amplicon length of 491 bp. To construct the quantitative PCR standards, the purified PCR products were ligated into the pMD 18-T Vector (Takara Biotech, Dalian, China), then transformed into the Top 10 chemically competent *E. coli* (Takara Biotech). The extracted and purified vectors were used as standards by serial dilution of the plasmids carrying the respective gene targets.

## 2.6 DNA sequencing

The PCR amplification of AOA and AOB *amoA* genes was undertaken on an ABI 9700 thermocycler (Thermo Fisher Scientific Inc.), using the same primers and cycling conditions as described above for quantitative PCR. Barcodes and linkers were added to each forward primer. The PCR products from different samples were quantified using the QuantiFluor<sup>TM</sup>-ST System (Promega, Wisconsin, US) and pooled at equal concentrations. Amplicon sequencing was performed on a pyrosequencing Roche GS FLX Titanium platform (454 Life Sciences, Branford, CT, USA) according to the manufacture's protocols.

## 2.7 Bioinformatics analyses

DNA raw sequence data were subjected to systematic checks using the following criteria: errors from PCR amplification and pyrosequencing were reduced using Usearch software (version 7.1) [29]. Briefly, reads quality was controlled using a sliding window approach: when the average quality score within a 50-bp window dropped below 20, the sequence was trimmed, sequences with mismatches to either the primer ( $> 2$ ), with one or more ambiguous bases or with homologs longer than 10 bp, or sequence lengths  $< 200$  bp were removed. The remaining high-quality sequences were screened using UCHIME [30] to discriminate and eliminate putative chimeras. After quality control, a total of 241,820 and 168,398 high quality raw sequence reads were obtained for AOA and AOB, respectively.

The valid sequences obtained were clustered into operational taxonomic units (OTUs) using Usearch software (version 7.1) with a sequence identity threshold of 97% for both AOA and AOB *amoA* gene. The duplicated sequences were removed using the `fastx_unique` command, and the singletons were discarded using the `sortbysize derep.fasta` command in Usearch. A total of 64 and 51 OTUs were obtained for AOA and AOB, respectively. The representation sequences of each OTU of the *amoA* gene were aligned against the Fungene database [31].

The diversity statistics and rarefaction were calculated subsequently. To eliminate the bias of libraries' alpha diversity comparison, the same number of sequences in each pyrosequencing library was subsampled randomly (6853 reads for AOA and 5230 reads for AOB) by Mothur [32] ([http://www.mothur.org/wiki/Schloss\\_SOP](http://www.mothur.org/wiki/Schloss_SOP)). Good's coverage [33] of AOA and AOB in all the 24 samples exceeded 99% (Table S1), revealing that these libraries could well reflect the diversity of

archaeal and bacterial *amoA* gene. Chao richness [34] and Shannon index [35] were calculated to describe the community's richness and diversity using Mothur [32]. The results of Chao richness and Shannon index of AOA and AOB *amoA* genes were listed in Table S3. phylogenetic trees were constructed based on the OTUs with relative abundance > 0.1% of AOA and AOB *amoA* gene, by the neighbor-joining method using a Kimur 2-parameter distance with 1,000 bootstrap replicates with MEGA 6 [36].

All original nucleotide sequence reads were deposited at the NCBI Sequence Read Archive (SRA) with the accession number of SRP091934.

## 2.8 Statistical analyses

One way analysis of variance (ANOVA) and correlation analysis was conducted using SAS 8.1, and the Duncan's test was employed to evaluate significance ( $p < 0.05$ ) in ANOVA. The relationship between green manure, soil properties, NP, abundances and communities of AOA and AOB were explored using partial least squares path modeling (PLS-PM). PLS-PM is a statistical method for studying complex multivariate relationships among observed and latent variables [37]. The data of AOA abundance and community at S1 stage and AOB at S1 stage were used to construct the model. Five latent variables were used: Green manure, soil properties (Soil), NP, abundance and communities of AOA and AOB. Carbon to nitrogen ratios of the green manures, N input, P input and K input from the green manures were selected as the manifest variables (i.e. observed variables) to reflect the latent variable green manure; pH, SOM, TN,  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$  and AK were selected as the manifest variables of soil properties; *amoA* gene copy numbers of AOA and AOB were the observed variables of AOA and AOB abundances, respectively; the

relative abundances of main OTUs of AOA and AOB (12 abundant OTUs for each library) were the observed variables to reflect the community structure of AOA and AOB, respectively. The goodness of fit (GoF) index was calculated as the geometric mean of the average communality and  $R^2$  value in the model and assess the overall prediction performance of the model [37]. The R package “plsrm” [38] was used to construct the model.

### 3 Results

#### 3.1 Response of soil chemical properties to green manuring

Some soil properties changed significantly following the application of green manures (Table 1). At Stage 1, 30 days after incorporation, RD decreased the total N and  $\text{NO}_3^-$ -N contents ( $p < 0.05$ ). Available K increased significantly independently of which green manure was applied ( $p < 0.05$ ). After the rice was harvested (Stage 2), green manure treatments had significantly lower pH values than WF ( $p < 0.05$ ). Compared with WF, MV decreased  $\text{NH}_4^+$ -N but increased the  $\text{NO}_3^-$ -N content; RD increased soil Total N and the available-P content; RG decreased  $\text{NH}_4^+$ -N and increased the available-P content ( $p < 0.05$ ). At Stage 2, all the soil chemical properties except pH and available P were lower than those at Stage 1 (Table 1).

#### 3.2 Response of the nitrification potential

Soil nitrification potential ranged from 11.75 to 51.54  $\text{ng N g}^{-1} \text{h}^{-1}$ , and was higher at Stage 1 than that at Stage 2. At Stage 1, the incorporation of radish increased the soil nitrification potential compared with WF and MV. After harvesting, there were no significant differences between treatments (Fig 1).

### 3.3 Quantification of archaeal and bacterial *amoA* genes and their correlations with soil properties

Archaeal *amoA* genes, as indicators for AOA, were always more abundant than those of bacteria, indicating the presence of AOB. The ratios of AOA to AOB *amoA* genes ranged from 7 to 80. MV increased both the AOA and AOB *amoA* genes (Fig. 2). For the AOA *amoA* gene, MV were 3.1 and 1.6 times higher than that in WF at Stage 1 and Stage 2, respectively. For the AOB *amoA* gene, MV were 6.5 and 3.7 times higher than WF at Stage 1 and Stage 2, respectively. RG also increased the AOA and AOB *amoA* gene at Stage 2 significantly, while RD had no effect on the abundance of both AOA and AOB *amoA* genes (Fig. 2).

Statistical analysis of the relationships between AOA and AOB *amoA* genes abundances and soil NP, pH,  $\text{NH}_4^+\text{-N}$  (Fig 3) demonstrated that abundances of AOA *amoA* gene correlated negatively with NP and  $\text{NH}_4^+\text{-N}$  ( $p < 0.05$ ), and positively with pH ( $p < 0.01$ ). No significant correlations were observed between the abundance of AOB *amoA* gene and NP, pH, and  $\text{NH}_4^+\text{-N}$ .

### 3.4 Diversity of *amoA* genes from archaea and bacteria

The most abundant OTUs (relative abundance higher than 0.1%) of *amoA* genes from archaea and bacteria were used to construct the phylogenetic trees. The results showed that the most abundant OTUs of AOA *amoA* were mainly affiliated with *Nitrososphaera* (group 1.1 b) and *Nitrosotalea* (group 1.1 a-associated) cluster (Fig 4A). The number of reads affiliated to the *Nitrososphaera* and *Nitrosotalea* clusters accounted for 85.0% and 14.3% of total reads, respectively. The archaeal *amoA* OTUs of *Nitrososphaera* were distributed among four subclusters.

Subcluster 1 included the most abundant sequences, accounting for 79.7% of the total sequences. The affiliation of the *amoA* sequences detected in this study to the four different subclusters and their abundances are shown in Fig 4B. RD was associated with clear changes in the composition of AOA *amoA* which significantly increased the relative abundance of the *Nitrosotalea* cluster (group 1.1a-associated), subcluster 2 and subcluster 3 of *Nitrososphaera*, and decreases in Subcluster 1 at both sampling stages compared with WF ( $p < 0.05$ ). The MV and RG treatments influenced the composition of AOA *amoA* by changing the relative abundance of some subclusters. MV significantly decreased the relative abundance of subcluster 2 of *Nitrososphaera* at Stage 1 and increased the subcluster 3 of *Nitrososphaera* at Stage 2 ( $p < 0.05$ ); RG significantly increased the subcluster 4 of *Nitrososphaera* at Stage 2 ( $p < 0.05$ ).

The main OTUs of AOB *amoA* were grouped into *Nitrospira* and *Nitrosomonas* clusters, accounting for 99.3% and 0.2% of all the sequences, respectively (Fig. 5A). Five subclusters of *Nitrospira* were found. They were Cluster 3a (0.3%), Cluster 3b (2.6%), Cluster 9 (2.7%) and Cluster 12 (91.0%), and another 2.6% of the sequences were clustered into *Nitrospira* but didn't belong to any of the known subclusters. Cluster 12 were the dominant cluster in all the treatments at both sampling stages. This cluster was not affected by the application of green manures (Fig 5B), while the abundance of other subclusters had minor variation after green manuring compared with WF. MV had a lower relative abundance of the unclassified cluster of *Nitrospira* at Stage 2; Radish had higher relative abundance in cluster 3a at Stage 1; Ryegrass had higher relative abundance in cluster 3b and lower relative abundance in the unclassified cluster of *Nitrospira* at Stage 2 ( $p < 0.05$ ) (Fig. 5B).

### 3.5 Partial least squares path modeling analysis

The PLS-PM model evaluated the correlations between green manure treatments, soil properties, NP, abundance and the communities of AOA (Fig 6A) and AOB (Fig 6B) at the first sampling stage. In the AOA model, green manure application had positive and soil properties had negative effects on the abundance of AOA, the path coefficients were 1.237 and -1.239, respectively. Nitrification potential was affected by the abundances and communities of ammonia oxidizers. In this model, the community of AOA had positive effects while their abundance had small negative effects on NP, indicating that the community of AOA might be the main factor that affect nitrification potential. In the model of AOB, soil properties had positive effects on the AOB community (the path coefficient was 0.842), both the abundance and community of AOB had no significant effects on nitrification potential.

The bi-group analysis was conducted between the two models to compare the differences of the structural coefficients, using the bootstrap t-test method with 1000 resamples to calculate the standard error estimates. The results of bi-group analysis showed that none of the path coefficients between AOA and AOB were significantly different, indicating that the green manure and soil properties had similar effects on AOA and AOB. The GoF index is a pseudo Goodness of fit measure to show the model quality at both the measurement and structural models [37]. The GoF of the two model in our study were 0.53 for AOA and 0.51 for AOB, respectively, suggesting that the overall prediction performance of the models were acceptable.

## 4 Discussion

AOA rather than AOB *amoA* gene were found to predominant in abundance at both sampling

stages in this study, i.e. 30 days after incorporation of green manure and after the rice was harvested. The soil we studied is a typical of acidic paddy soils in southern China. Many studies reported that AOA had a competitive advantage over AOB in acidic soils [3, 5, 39], and that AOA were better adapted to the alternation between oxygenated and hypoxic conditions [15, 40]. Some studies showed that the abundance and community of AOB were changed by inorganic nitrogen fertilizer such as ammonium based fertilizers or urea [11, 41]. By contrast, AOA abundance and community structure has been shown to change due to the application of the organic substrates and the combined application of organic and chemical fertilizers [11, 42]. In our study, AOA *amoA* was more sensitive to the application of green manures than AOB *amoA*, which is consistent with former studies [14, 41]. The utilization of green manure in paddy soils is an effective and valuable management approach to maintaining the sustainable development of agriculture. The decomposition of green manures following their incorporation releases large quantities of nitrogen and carbon [43]. The mineralized N released by green manures could be considered similar to that provided by organic fertilizer, and might favor AOA and promote their growth and activity. The greater abundance and sensitivity of AOA *amoA* to the application of green manures indicated that AOA might play more important roles in winter green manure-rice cropping system than AOB.

Nitrification potential may depend not only on population size but also on community composition. The change of communities structures of ammonia oxidizers may also lead to different responses in nitrification potential [12]. Our results suggest that AOA rather than AOB controlled nitrification in this system. The predominance of AOA in red paddy soils may be partly because of the acidic soil environment in which different green manures led to the differential responses of ammonia oxidizers and nitrification potential.



Paddy soil provides a complex environment for ammonia oxidizers. Within this environment, soil conditions such as temperature [44], pH [4, 5], nutrient content [12] and their interactions have various influences on AOA and AOB. The growth of green manures and decomposition of plant residues after incorporation adds significant quantities of organic C and N into soil. The nitrogen mineralized from incorporated plants is one of the substrates in nitrification and might stimulate the growth of ammonia oxidizing prokaryotes. The C/N of milk vetch, radish and ryegrass used in this study were 11.72, 14.89 and 16.83, respectively, and litter quality of these three green manures were different. Plant litter quality is an important factor regulating decomposition and nutrient cycling [45]. The different mineralization characteristics of these three green manures resulted in various effects on ammonia oxidizers. Application of milk vetch increased the abundance of AOA and AOB *amoA* genes, while radish increased the nitrification potential and changed the distribution of dominant groups in AOA *amoA*. These results suggest that green manures had different effects on nitrification and ammonia oxidizing prokaryotes, and the difference may be because of the different characteristics of the green manure crops.

The dominant groups in our study were *Nitrososphaera* of AOA and *Nitrosospira* of AOB, which are commonly detected in many soil environments [4, 17, 46]. Previous studies have recognized the *Nitrososphaera* cluster as the most abundant soil AOA lineage in both acidic and alkaline soils [42]. The *Nitrososphaera* cluster was detected with a strong genetic capacity to utilize various ammonia sources and was directly linked with nitrification activity in agricultural soils [16, 47, 48]. The *Nitrosotalea* cluster was also detected in our study. It was defined as an obligate acidophilic AOA, and provides a capacity for nitrification in acidic soils [49]. The existence of *Nitrosotalea* in our acidic red paddy soil was confirmed with these studies. For AOB,

*Nitrospira* were dominant in relatively low N environments, while *Nitrosomonas* were prevalent in environments with high N loadings [46]. Our study observed that most sequences of AOB were affiliated with the *Nitrospira* cluster 12. These findings are consistent with previous studies that identify that AOB are dominated by *Nitrospira* cluster 12 in acid red paddy soils, and the AOB distribution was not affected by different fertilization treatments [23].

In conclusion this study suggested that AOA and AOB have a quantitatively different importance in red paddy soil and that mainly AOA respond to green manuring. These results support emerging evidence of different ecological niche preferences of AOA and AOB in soils.

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501 **Table 1. Soil chemical properties in different treatments at different growth stages**

Treat	pH	SOM	TN	NH <sub>4</sub> <sup>+</sup> -N	NO <sub>3</sub> <sup>-</sup> -N	Available P	Available K
ment		(g kg <sup>-1</sup> )	(g kg <sup>-1</sup> )	(mg kg <sup>-1</sup> )	(mg kg <sup>-1</sup> )	(mg kg <sup>-1</sup> )	(mg kg <sup>-1</sup> )
<i>30 days after green manure incorporated (S1)</i>							
MV	5.12 a	40.9 a	1.83 a	31.2 a	88 a	94 a	169 b
RD	5.12 a	41.6 a	1.77 b	41.0 a	77 a	97 a	194 a
RG	5.18 a	39.6 a	1.81 a	28.0 a	55 b	98 a	208 a
WF	5.04 a	34.0 a	1.85 a	29.3 a	84 a	96 a	131 c
<i>After rice harvesting (S2)</i>							
MV	5.56 b	34.2 a	1.70 b	11.6 b	26 a	123 a	28 b
RD	5.52 b	31.0 a	1.77 a	18.4 ab	15 ab	124 a	29 b
RG	5.57 b	29.0 a	1.72 ab	12.4 b	18 ab	120 ab	40 a
WF	5.76 a	29.0 a	1.69 b	23.0 a	8 b	111 b	26 b

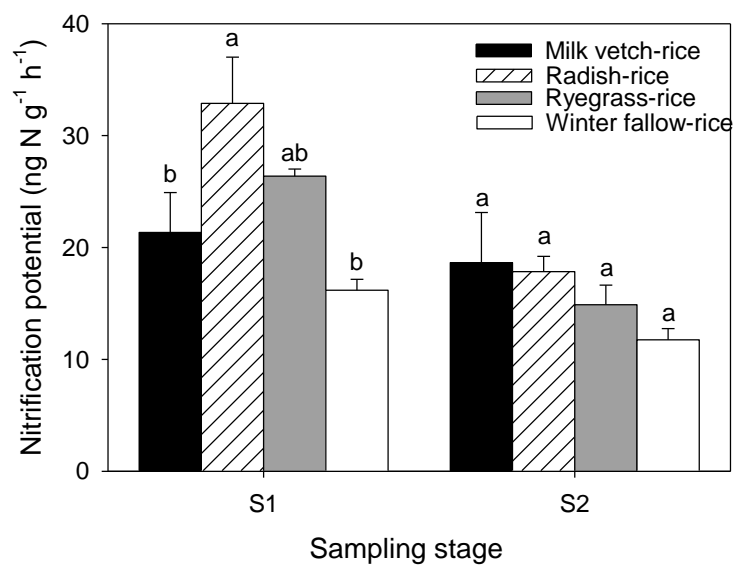
502 MV: milk vetch-rice; RD: radish-rice; RG: ryegrass-rice; WF: winter fallow-rice. Values are means ±SE (n=3).

503 Means followed by different letters are significantly different ( $p < 0.05$ ).

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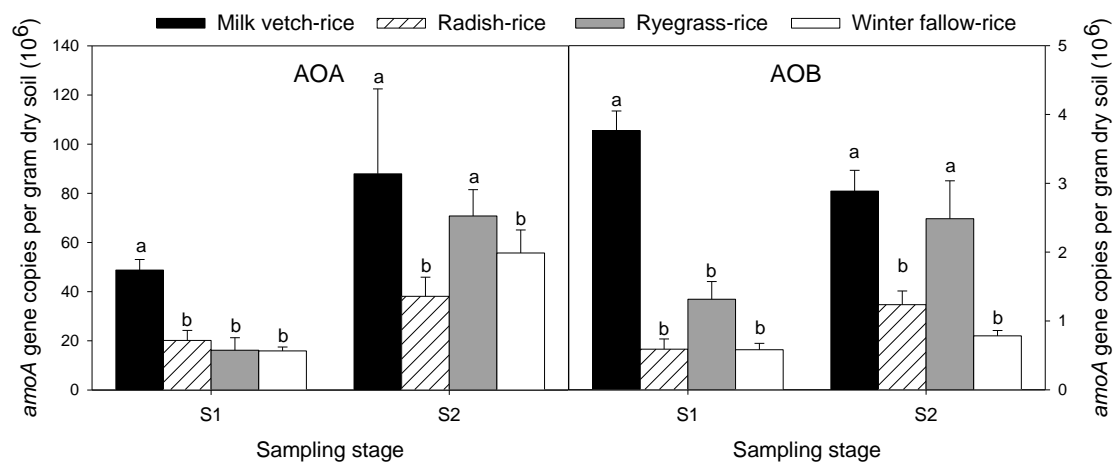


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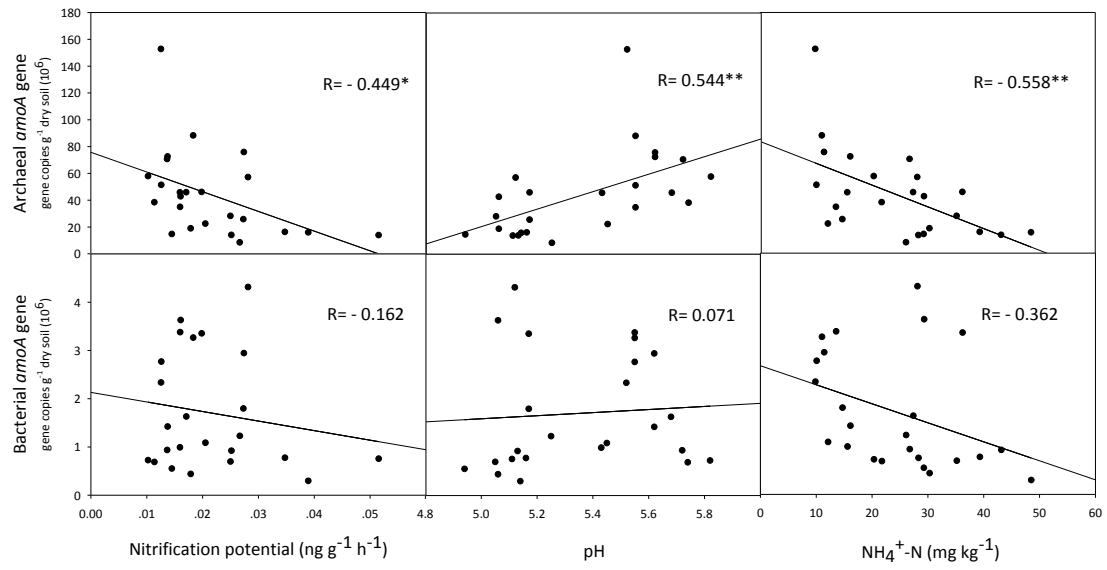
507 **Fig 1. Soil nitrification potential in different treatments at the two sampling stages. Vertical T bars indicate**

508 **SE. Bars topped by different letters are significantly different ( $p < 0.05$ ).**

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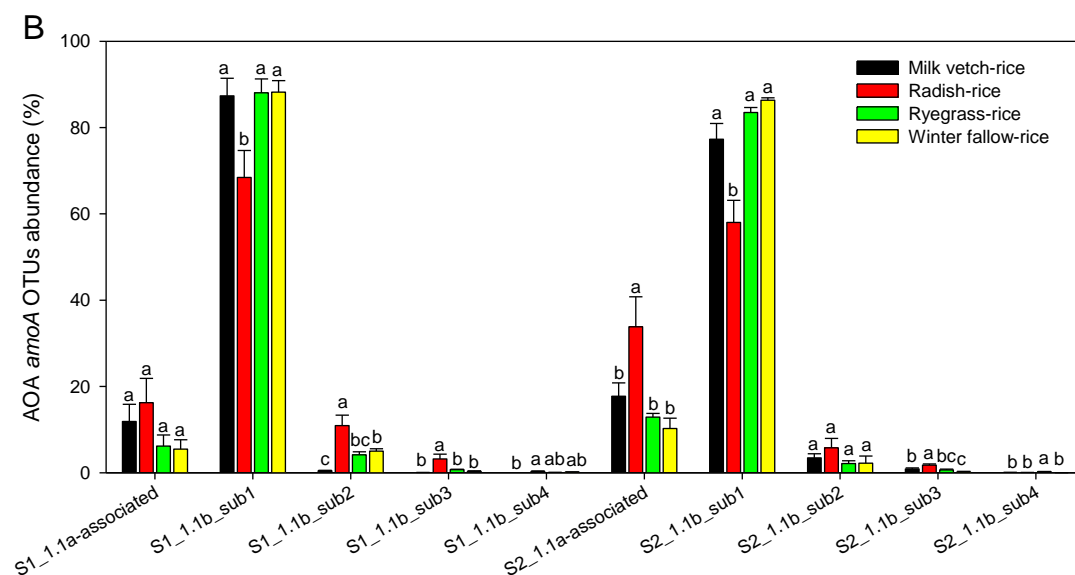
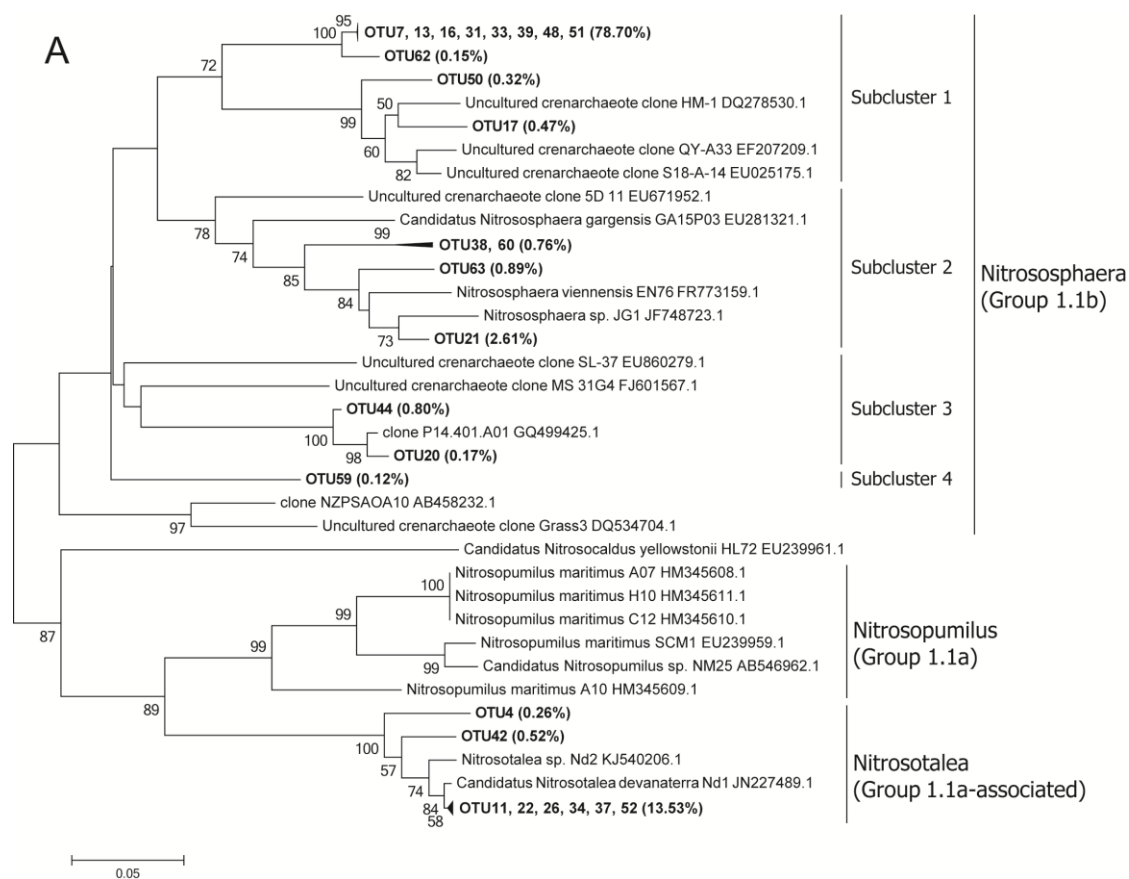


**Fig 2. Ammonia-oxidizing archaeal *amoA* abundance (AOA) and Ammonia-oxidizing bacteria *amoA* abundance (AOB) in different treatments at two sampling stages. Vertical T bars indicate SE. Bars topped by different letters are significantly different ( $p < 0.05$ ).**

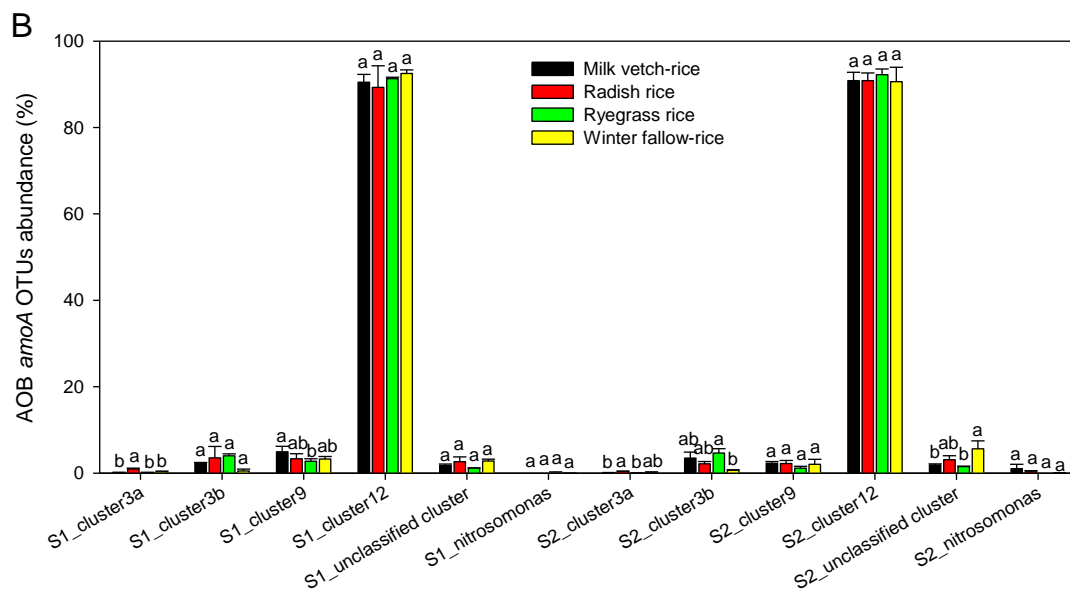
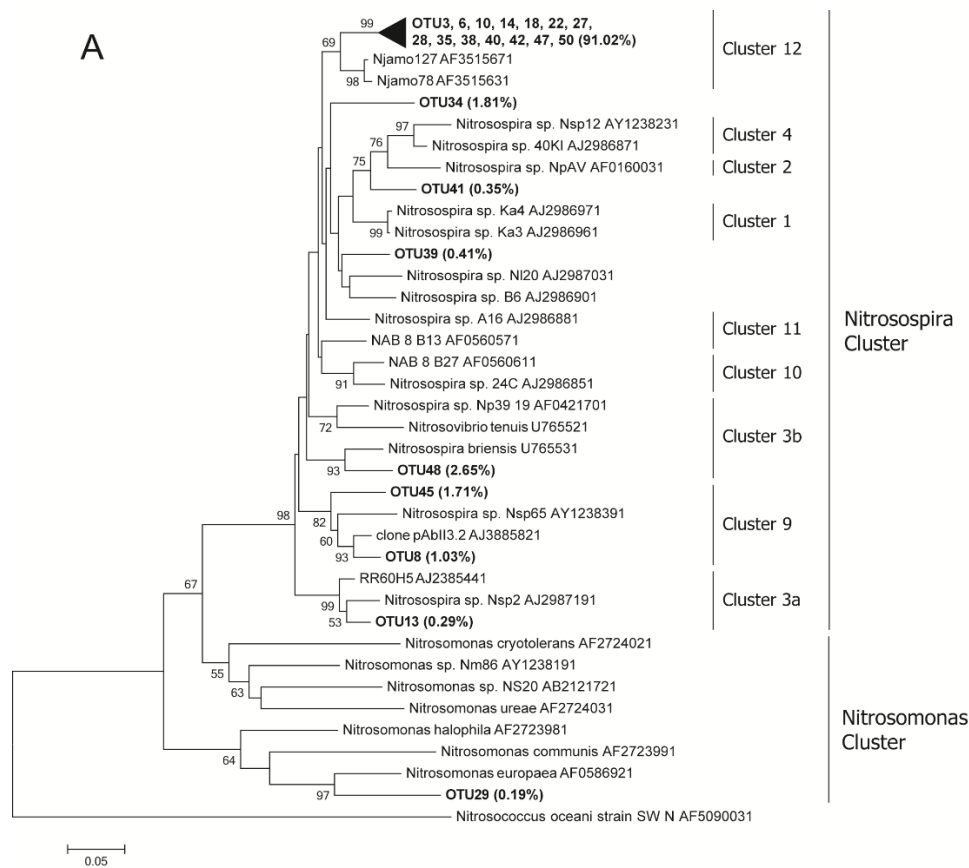


**Fig 3. Relationships between archaeal and bacterial *amoA* gene abundances and soil NP, pH, NH<sub>4</sub><sup>+</sup>-N. \*  $p <$**

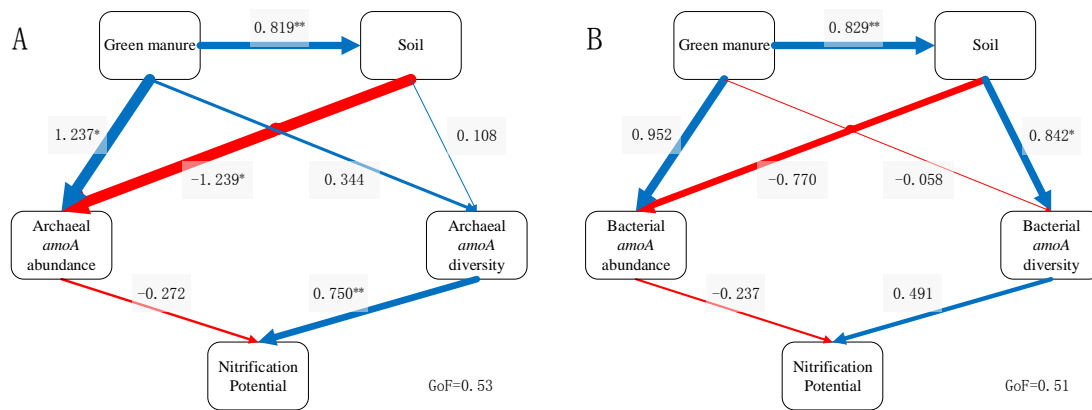
**0.05, \*\*  $p < 0.01$ , n=24.**



**Fig 4. A)** A Neighbor joining tree for AOA partial *amoA* OTUs (representatives with relative abundance > 0.1%). AOA *amoA* OTUs in this study are shown in bold. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values of > 50% are showed at branch points. **B)** The composition analysis of AOA *amoA* gene in the four treatments at the two sampling stages.



**Fig 5. A) A neighbor joining tree for AOB partial *amoA* OTUs (representatives with relative abundance > 0.1%). AOB *amoA* OTUs in this study are shown in bold. The scale bar represents 5% nucleic acid sequence divergence, and bootstrap values of > 50% are showed at branch points. B) The composition analysis of AOB *amoA* gene in the four treatments at the two sampling stages.**



**Fig 6. Directed representation of the Partial Least Squares Path Model (PLS-PM). AOA at Stage 1 (A) and AOB at Stage 1 (B) were analyzed separately. Only latent variables were represented in the graph. Green manures represented the different treatments. Soil represented soil properties. Archaeal *amoA* diversity and Bacterial *amoA* diversity represented the distribution of AOA *amoA* OTUs and AOB *amoA* OTUs. Indicated value are the path coefficients. Larger path coefficients were reflected in the width of the arrow; blue arrow indicated a positive effect, while red arrow indicated a negative effect. Path coefficients that differ significantly from 0 were indicated by \*  $p < 0.05$  and \*\*  $p < 0.01$ . Significance was based on 1000 resampled bootstraps. GoF indicated the Goodness of Fit, a measure of the overall prediction.**